INFECTION AND IMMUNITY, Jan. 2006, p. 273–281 Vol. 74, No. 1
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.1.273–281.2006

Interaction with CagF Is Required for Translocation of CagA into the Host via the *Helicobacter pylori* Type IV Secretion System

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Received 22 July 2005/Returned for modification 17 August 2005/Accepted 14 October 2005

Development of severe gastric diseases is strongly associated with those strains of *Helicobacter pylori* that contain the *cag* pathogenicity island (PAI) inserted into the chromosome. The *cag* PAI encodes a type IV secretion system that translocates the major disease-associated virulence protein, CagA, into the host epithelial cell. CagA then affects host signaling pathways, leading to cell elongations and inflammation. Since the precise mechanism by which the CagA toxin is translocated by the type IV secretion system remained elusive, we used fusion proteins and immunoprecipitation studies to identify CagA-interacting secretion components. Here we demonstrate that CagA, in addition to other yet-unidentified proteins, interacts with CagF, presumably at the inner bacterial membrane. This interaction is required for CagA translocation, since an isogenic nonpolar *cagF* mutant was translocation deficient. Our results suggest that CagF may be a protein with unique chaperone-like function that is involved in the early steps of CagA recognition and delivery into the type IV secretion channel.

Helicobacter pylori is a gram-negative, microaerophilic, spiral-shaped bacterium that colonizes the gastric epithelium of the human stomach. *H. pylori* has been implicated in significant gastric maladies, such as peptic ulcer disease, chronic gastritis, mucosa-associated lymphoid tissue lymphoma, and adenocarcinoma (33, 37, 38, 41, 51, 55). Strains of *H. pylori* that are typically associated with these severe maladies in infected patients contain an intact *cag* pathogenicity island (PAI) that confers inflammation and ulceration in stomach cells (13, 57).

The *cag* PAI of *H. pylori* is a 40-kbp chromosomal region that was acquired by horizontal transfer and inserted at the distal end of the glutamate racemase gene (*glr*). Depending on the clinical strain, *cag* is comprised of 27 to 31 genes (1, 2, 13). A portion of these genes encode a type IV secretion system (T4SS) and a single known effector molecule, CagA (13). T4SSs are ancestrally related to conjugation systems and can be grouped according to their function into three categories: (i) DNA transfer (best characterized system for DNA transfer between gram-negative bacteria), (ii) DNA uptake and release, and (iii) effector translocation (11, 17, 27, 28, 30, 58). The T4SS of *H. pylori* belongs to the effector translocator group of T4SSs. This group contains several prominent plant and animal pathogens that deliver effector molecules to their target eukaryotic cell via what is thought to be a needlelike surface organelle. Other examples include *Agrobacterium tumefaciens*, *Brucella suis*, *Bartonella henselae*, and *Legionella pneumophila* (11, 14, 58).

The VirB/D4 type IV secretion system of *Agrobacterium tumefaciens* has become the standard reference of comparison for effector translocation T4SSs in gram-negative bacteria. Most known T4SSs in bacterial pathogens are encoded by genes showing ancestral relation to the genes encoding the VirB/D4 apparatus in *A. tumefaciens*. *Helicobacter pylori* possesses orthologs to all the VirB/D4 transport system proteins except VirB6 and the pilus subunits VirB2 and VirB5 (2, 10, 13, 29). Furthermore, the eight known VirB/D4 orthologs in *H. pylori* are absolutely required for translocation of CagA, along with 10 additional Cag proteins for which there is no detectable sequence similarity in the database (20). As well, there is no evidence for vertical descent for the *H. pylori* T4SS or any other type IV protein transporter system. A likely result of this nonvertical decent is the presence of the additional accessory genes in the *H. pylori* T4SS. Therefore, in-depth molecular analysis of the system, rather than functional inference from the VirB/D4 homologues of *A. tumefaciens*, is necessary to further our knowledge of this complex system.

To date, considerable attention has been given to the CagA effector protein, expanding our understanding of its function in the eukaryotic cell; however, the specific mechanism by which *H. pylori* translocates CagA into the host gastric epithelial cell is largely unknown. CagA remains the only identified effector that is translocated by the T4SS of *H. pylori* into gastric epithelial cells (4, 37, 46, 50). Following translocation, CagA is phosphorylated on multiple EPIYA motifs by Src family kinases and recruited to the plasma membrane, where it interacts with various host cell proteins, including SHP-2 phosphatase (3, 6, 26, 49, 54). Interaction of the SH2 domain of SHP-2 with tyrosine-phosphorylated CagA activates SHP-2 phosphatase activity and induces mitogen-activated protein kinase/MEK/extracellular signal-related kinase (ERK) signaling pathways, leading to abnormal proliferation of gastric epithelial cells and cell scattering, otherwise known as the "hummingbird phenotype" (43). Other substrates which CagA interacts with include ZO-1, Grb2, c-Met, and CSK (3, 9, 15, 31, 54, 59). With the exception of the VirB/D4 orthologs, the functions of the remaining proteins have proved elusive based on the lack of similarity to known proteins. To determine the mechanism of CagA recognition and translocation by the T4SS, we attempted to identify proteins that could potentially interact with CagA.

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TABLE 1. Oligonucleotides used in this study

Gene/target DNA	Function	Forward and reverse primers
cagA/HP0547	Antigenic effector molecule, secreted by T4SS	TATCTCGAGCATATGACTAACGAAACCATTAACCA TATTCTAGAAGATTTTTGGAAACCACCTTTTG
$cagA^{PR}$	Promotor of CagA antigen	TATCTCGAGTAGAACGCTTCATGCACTCACC TTTCATATGTTCTCCTTACTATACCTAGTT
cagF/HP0543	CagA translocation	TATATATCATATGAAACAAAATTTGCGTGAAC TATTCTAGAATCGTTATTTTTGTTTTGATTTTT
recA (down)	Site-specific recombinase activity	AAGCGGCCGCGATGACCCTTTAGAAGAAATGGA AAGAGCTCGCCACTAGCCATGCTTAAACAAC
recA (up)	Site-specific recombinase activity	AAGGTACCGGTTTGTGGCGAGCGTGCGTTTG AACTCGAGCTTATCCCCAAGGCGCACCAACG
orf8-FLAG/ HP0522	Interleukin-8 induction, CagA translocation	ATAATACATATGTTTAGAAAACTAGCAACC ATAGGATCCTCACTTGTCATCGTCGTCCTTGTAATCTCTAGACTTTGAATCTTTCAGTAACGC
cagF (up)	Homologous region upstream of *cagF*	TATGGTACCCTTGCGACTCAAAGCATCACTGAT TTAATCTCGAGTTGTTCACGCAAATTTTGTTTCAT
cagF (down)	Homologous region downstream of *cagF*	AAAATCTAGAGATGAAGTAACAAACAAAATGCTC ATATATATGCGGCCGCTGCAACACCACTTGTGCTTGAGAT

Here we describe the first interaction between the effector protein CagA and another *cag*-encoded protein, CagF (Hp0543). In contrast to a previous report (48), we demonstrate that CagF probably localizes to both the inner bacterial membrane and the cytoplasm and may be a protein with a unique chaperone-like function that recruits CagA to the T4SS.

MATERIALS AND METHODS

PCR and plasmids. All oligonucleotides used for PCR amplification are shown in Table 1. Plasmids used in this work are described in Table 2. Plasmids were constructed to express epitope-tagged versions of Cag proteins in *Escherichia coli* and *H. pylori*. The plasmid pBluescript SK(+) (Stratagene) was modified to yield the plasmid pSK+recx*orf*8. Briefly, a 1,500-base-pair region upstream of the recombinase gene in *H. pylori* (*recA*) was amplified using the oligonucleotides recUp+ and recUp− and cloned into the KpnI/XhoI sites of the pBluescript SK(+) vector. The resulting vector was digested with NotI/SacI, and a 750-base-pair *recA* downstream region was inserted, which was amplified using the oligonucleotides recD+ and recD− and digested with identical enzymes. A kanamycin resistance gene cassette (*kan*) was introduced between the *recA* upstream and downstream regions using the BamHI and NotI restriction sites. The *cagA* promoter was amplified ($CagA^{Pr}$+ and $CagA^{Pr}$−) and digested by XhoI/NdeI. *orf8* was amplified (orf8FL+ and orf8FL−), introducing a FLAG sequence flanked by XbaI and BamHI restriction sites at the 3′ end of the gene. Both fragments, the *cagA* promoter and *orf8*-FLAG, were simultaneously inserted between the *kan* cassette and the *recA* upstream region (Xho/BamHI). The resulting vector was designated pSK+recx*orf*8. To create pSK+recx*cag*A and pSK+recx*cag*F, the *orf8* gene was excised with NdeI/XbaI from the vector and replaced by one of the respective genes, which were amplified excluding the stop codons, using the oligonucleotide pairs cagA+ and cagA− and cagF+ and cagF−.

To express the CagF-FLAG fusion from pACYC184 (New England Biolabs), *cagF*-FLAG including the *cagA* promoter was excised by using XhoI/BamHI from pSK+recx*cagF* and cloned into pACYC184 digested with SalI/BamHI. To express CagA without the FLAG tag, *cagA* was amplified with its natural promoter using the oligonucleotides $cagA^{Pr}$+ and cagAD− and cloned into pBluescript SK(+) using the SalI/NotI sites.

Bacterial strains and growth conditions. All *H. pylori* strains (Table 2) were grown on brucella broth medium supplemented with 5% fetal bovine serum (FBS), and the following antibiotics were added for selection: trimethoprim (10 μg/ml), vancomycin (5 μg/ml), amphotericin B (8 μg/ml), and cycloheximide (100 μg/ml). Strains G27/8-FL, G27/F-FL, G27/A-FL, and G27Δ*cagF*/F-FL were grown on the brucella broth plates described above supplemented with kanamycin (20 μg/ml). Incubation of *H. pylori* strains was performed at 37°C for 48 h in an anaerobic jar containing 5% CO_2, 5% H_2, and 90% N_2. Liquid cultures were grown in brucella broth supplemented with 10% FBS (previous antibiotics and concentrations) in an anaerobic jar containing a Campygen gas mix of 5% O_2, 10% CO_2, and 85% N_2 (Oxoid). Cultures were grown at 37°C overnight, rotating at 160 rpm. *E. coli* strains (Table 2) were grown on LB plates and medium supplemented with the appropriate antibiotics: kanamycin (50 μg/ml), chloramphenicol (50 μg/ml), and ampicillin (100 μg/ml).

Helicobacter pylori strain G27 has been described previously (57). The G27 *cagF*-deleted mutant G27Δ*cagF*/F-FL was generated as described previously by Copass et al. (15a). Briefly, short regions of the *cagF* 5′ (oligonucleotides FUp+ and FUp−) or 3′ end (oligonucleotides FD+ and FD−) containing several hundred base pairs of flanking sequences were amplified by PCR and succes-

TABLE 2. Strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli		
DH5α		Invitrogen
DH5αAF	Cm^r Ap^r; pSK+*cagA* and pACYC184 × *cagF*	This study
DH5αA	Ap^r; pSK+*cagA*	This study
H. pylori		
G27	Wild-type clinical isolate strain	Xiang et al., 1995 (57)
G27/A-FL	Kan^r, $cagA^{PR}$-*cagA*-FLAG-kanamycin resistance cassette inserted at *recA*	This study
G27/F-FL	Kan^r, $cagA^{PR}$-*cagF*-FLAG-kanamycin resistance cassette inserted at *recA*	This study
G27/8-FL	Kan^r, $cagA^{PR}$-*orf8*-FLAG-kanamycin resistance cassette inserted at *recA*	
G27Δ*cagA*	Kan^r	This study
G27Δ*cagF*		This study
G27Δ*cagF*/F-FL	Kan^r, insertion at *recA*	This study
G27Δ*virB9*		This study
Plasmids		
pSK+recx*orf*8	Km^r Ap^r; vector for *H. pylori* transformation of *orf8*-FLAG	This study
pSK+recx*cag*A	Km^r Ap^r; vector for *H. pylori* transformation of *cagA*-FLAG	This study
pSK+recx*cag*F	Km^r Ap^r; vector for *H. pylori* transformation of *cagF*-FLAG	This study
pACYC184	Tc^r Cm^r	New England Biolabs
pACYC184 × *cagF*	Cm^r vector for expression of CagF-FLAG in *E. coli* under control of $cagA^{PR}$ (insertion of gene product at XhoI and BamHI restriction sites)	This study
pSK+*cagA*	Ap^r; $cagA^{PR}$ and *cagA* gene (insertion of gene product at XhoI and XbaI restriction sites)	This study
pMA1	pSK + vector with CagF up- and downstream sequences (upstream region cloned into KpnI and XhoI, downstream cloned into XbaI and NotI)	This study
pMA2	pMA1; Km^r and *sacB* insertion (insertion between up- and downstream regions of *cagF* at XhoI and XbaI restriction sites)	This study

sively cloned after digestion into the pBluescript SK(+) vector to create pMA1. A cassette containing a kanamycin resistance gene and the *sacB* gene was then cloned between the two amplified regions, resulting in vector pMA2. Following transformation of pMS1 into strain G27, the *cagF-kan* mutant was selected for kanamycin resistance and the corresponding DNA region was amplified by PCR to confirm recombination. To create a nonpolar *cagF* deletion strain that was lacking artificially introduced genes, the *cagF-kan* mutant was transformed with pMA2 and mutants selected on brucella broth plates containing 10% sucrose. Colonies were confirmed by PCR and by testing for sensitivity to kanamycin.

***H. pylori* transformation.** To express a FLAG-tagged Orf8, CagA, and CagF protein in G27, the vectors pSK+recx*orf*8, pSK+recx*cag*A, and pSK+recx*cag*F were transformed into the *H. pylori* strain G27 or the Δ*cagF* mutant, and recombinants expressing the FLAG-tagged proteins were selected on brucella broth plates containing 20 μg/ml kanamycin. Replacement of the *recA* gene by the individual FLAG-tagged *cag* genes via homologous recombination was confirmed by PCR and immunoblotting.

Immunoblotting. Samples were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to Immobilon-P polyvinylidene difluoride (PVDF) (Millipore) membranes, and blocked in 5% (wt/vol) skim milk. All primary and secondary antibodies were diluted in 1% (wt/vol) bovine serum albumin. FLAG-tagged proteins were detected with a primary monoclonal mouse anti-FLAG antibody (1:5,000) (Sigma). CagA was detected with a polyclonal rabbit anti-CagA serum (1:2,500) (kindly provided by Antonello Covacci). CagF was detected with precleared anti-CagF rabbit polyclonal serum (48). The polyclonal rabbit antibody anti-VirB11 (1:5,000) (kindly provided by Rainer Haas) was used to detect the putative inner membrane protein VirB11 (Hp0525). The mouse monoclonal antibody anti-HopE (1:1,000) (kindly provided by Peter Doig) was used to detect the outer membrane protein HopE. Secondary antibodies were anti-rabbit or anti-mouse conjugated to horseradish peroxidase (Sigma), and membranes were developed using the chemiluminescent substrate ECL (Amersham).

^{35}S metabolic labeling, cross-linking, and immunoprecipitation. *Helicobacter pylori* strains were grown under microaerophilic conditions overnight in 90% RPMI-based minimal medium (lacking Cys and Met)–10% brucella broth mixture at 37°C shaking at 160 rpm. Ten microliters of Redivue [^{35}S]methionine (Amersham) was added to each culture and incubated for an additional 5 h. Three milliliters of each culture was collected by centrifugation, washed two times, and resuspended in 200 μl 10 mM phosphate-buffered saline. Ten microliters of 10% NP-40 (Calbiochem) and then 15 μl of 25 mM dithiobis (succinimidyl propionate) (DSP) in dimethyl sulfoxide was added to each sample. Samples were incubated on ice for 2 h, and the cross-linking reaction was stopped with 5 μl of 1 M Tris (pH 7.5). For immunoprecipitation, a threefold volume of lysis buffer (150 mM phosphate-buffered saline, 7.15% [wt/vol] sucrose, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1/66 of 15 μg/ml) was added to each sample. Cells were frozen and thawed 5 times and centrifuged for 10 min at 18,000 × *g*. The supernatant was removed and added to 30 μl anti-FLAG M2 agarose beads (Sigma). Beads were previously equilibrated with three washes of lysis buffer and blocked with 1% (wt/vol) bovine serum albumin for 6 h to eliminate nonspecific binding. The supernatant and beads were incubated at 4°C for 2 h on a rotary shaker. Beads were centrifuged at 16,100 × *g*, washed in lysis buffer three times, resuspended in 50 μl of 1× Laemmli sample buffer (5% β-merceptoethanol), and boiled for 10 min.

Bacterial fractionation via differential centrifugation and detergent solubility. Bacterial fractionation was performed as described previously by Gauthier et al. (25). *H. pylori* strains were grown overnight in brucella broth medium and adjusted to an approximate optical density at 600 nm of 1.5. Cells were sonicated five times for 1-min bursts at 35% output (Fisher sonic dismembrator; amplitude, 1.4). *N*-Lauroyl sarcosine was used at 2% (wt/vol) for solubility of the inner membrane proteins with rotation at room temperature for 30 min. The periplasmic and cytosolic fractions were recovered in 1 ml, the inner membrane fraction in 500 μl, and the outer membrane in 100 μl of each appropriate buffer. Equal percentages of each sample were analyzed by immunoblot analysis.

Infection and fractionation of AGS cells. Gastric epithelial AGS tissue culture cells (ATCC CRL-1739) were grown at 37°C in 5% CO_2–95% air in RPMI medium supplemented with 10% (vol/vol) FBS (Invitrogen). In vitro infection and fractionation of AGS cells yielding cytosolic, membrane, and insoluble/bacterial fractions were performed as described previously by Stein et al. (50). AGS cells were infected at a multiplicity of infection of 100:1.

RESULTS

CagF interacts directly with two molecular mass species of the CagA antigen in *E. coli*. We sought to screen for *cag* protein interactions using an activity reconstitution-based bacterial two-hybrid system, because a yeast two-hybrid assay did not identify any interactions between members of the *cag* PAI

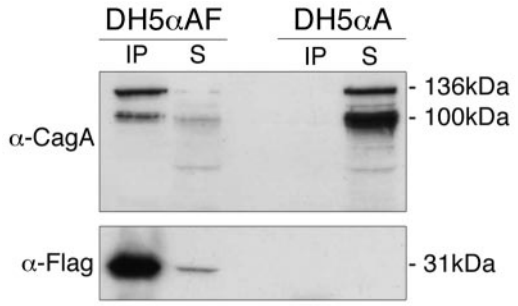

FIG. 1. CagA coimmunoprecipitates with CagF after expression in *E. coli*. CagA was coexpressed with CagF in *E. coli* strain DH5α (DH5αAF) or expressed alone as a negative control (DH5αA). Proteins interacting with CagF were coimmunoprecipitated from cell lysates with monoclonal anti-FLAG antibody coupled to agarose beads. Immunoprecipitates (IP) and supernatants (S) were separated by 9% SDS-PAGE and transferred onto PVDF membranes in duplicate. The membranes were probed with polyclonal CagA antiserum (α-CagA) or with monoclonal anti-FLAG antibody (α-FLAG). Blots were developed with peroxidase-coupled secondary antibodies.

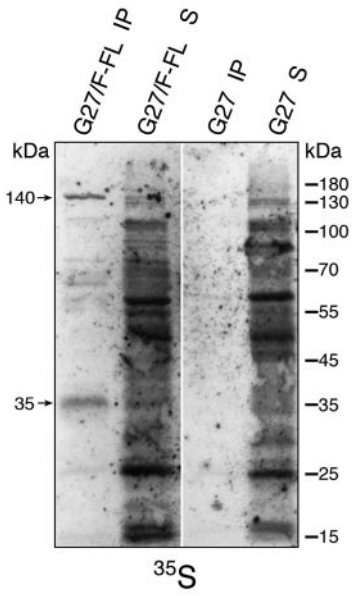

FIG. 2. Immunoprecipitation (IP) of ^{35}S-labeled proteins that interact with CagF in *H. pylori*. *H. pylori* strains G27 (wild type) and G27/F-FL were metabolically labeled with ^{35}S for 5 h. Lysis, fractionation, and immunoprecipitation of both strains were performed as described for Fig. 1. Following 10% SDS-PAGE and transfer onto PVDF membranes, the ^{35}S-labeled proteins in each sample were visualized by exposure of the membrane to X-ray film.

(53). We identified several potential interacting partners but were unable to obtain reliable and consistent results (unpublished data). One possible interaction revealed by the bacterial two-hybrid results involved the CagA antigen and a protein of unknown function, CagF. To test this potential interaction, plasmids expressing FLAG-tagged CagF and native CagA were constructed as described and cotransformed into *E. coli*. Coimmunoprecipitation of CagA together with the FLAG-tagged CagF in *E. coli* supported the interaction between the two proteins (Fig. 1). CagA was not unspecifically precipitated by the FLAG beads when expressed alone, supporting the specificity of the CagA-CagF interaction. The ability for the two proteins to interact in *E. coli* suggests that the interaction occurs directly and is not conferred by an intermediary Cag protein. CagA is unstable in *E. coli* and breaks down into two major fragments, a 100-kDa amino-terminal fragment and a 35-kDa carboxy-terminal species as seen with *H. pylori* (32, 36, 57). Both the full-length and cleaved 100-kDa fragment of CagA coprecipitated with CagF. These results suggest that the distal carboxy-terminal portion of CagA may affect, but is not absolutely required for, CagF binding.

Confirmation of the CagA-CagF interaction in *H. pylori*. A chromosomal insertion of CagF-FLAG was made in strain G27 as described in Materials and Methods. *H. pylori* strains G27 and G27/F-FL were metabolically labeled with ^{35}S and cross-linked with DSP, and cell lysates were applied to anti-FLAG agarose beads (Sigma). Samples of the immunoprecipitations and supernatants were subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were developed by autoradiography (Fig. 2). An intense band running in the 35-kDa range was detected on the radiograph. This band comigrated with the specific immunoprecipitated CagF-FLAG (theoretical molecular mass, 32 kDa) and was absent in the control G27 immunoprecipitation. A second band in the G27/F-FL immunoprecipitation was present at roughly the 140-kDa range. This protein we suspected was CagA, and it was also not present in the G27 control precipitation. CagF was positively identified using the specific anti-FLAG antibody. CagA was positively identified using a polyclonal antibody directed against the full-length protein (data not shown). The lack of discernible immunoprecipitation bands with the G27 control strain suggests that the interaction with CagF was specific and that the proteins visualized were in fact coprecipitated. Several other bands were detected by autoradiographic analysis of the immunoprecipitate of G27/F-FL, suggesting that other proteins may be involved in a multiprotein complex. Further investigations of the identities of these proteins are under way in our laboratory.

The experiment was then modified to determine if this interaction was strong enough to be detected in the absence of the DSP cross-linker. Figure 3A shows that both CagA and CagF readily interacted in the absence of a cross-linking agent, suggesting that this interaction is specific, strong enough to allow coimmunoprecipitation, and not artificially induced by the cross-linker. CagA was also FLAG tagged and chromosomally inserted to allow for the reciprocal immunoprecipitation to be screened. The reciprocal immunoprecipitation of CagF with CagA-FLAG is illustrated in Fig. 3B. When FLAG-tagged CagA was immunoprecipitated from *H. pylori*, CagF was detected with a rabbit polyclonal CagF antibody. In order to ensure that the interaction was due to a specific protein interaction and not conferred directly by the FLAG tag fused to the C terminus of either CagF or CagA, Orf8, a *cag* protein from the distal 3′ region of the *cag* PAI, was FLAG tagged and used for immunoprecipitation as described above (Fig. 3C.). CagA was not detected, suggesting that the interaction between CagA and CagF was not an artifact caused by the presence of the FLAG tag. The G27 negative control was clear of

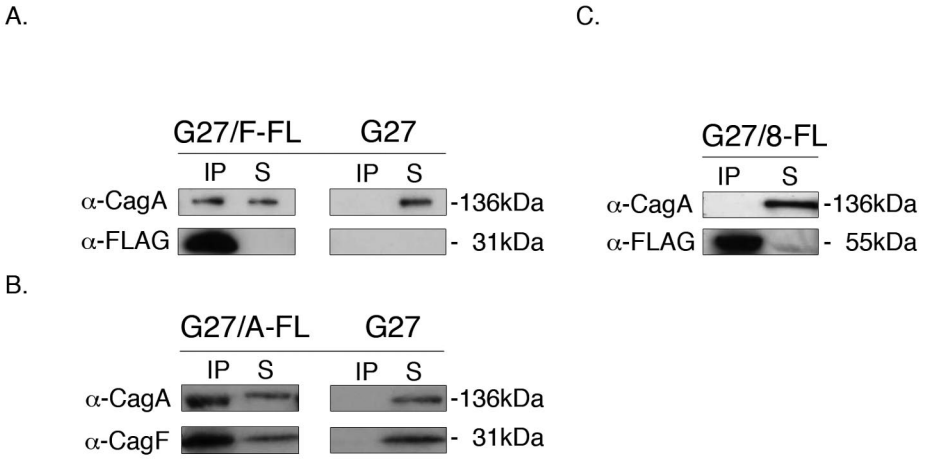

FIG. 3. CagA and CagF coimmunoprecipitate after expression in *H. pylori*. Lysates of *H. pylori* strains G27 (A and B), G27/F-FL (A), G27/A-FL (B), and G27/8-FL (C) were used in immunoprecipitation studies as described in the legend to Fig. 1. PVDF membranes were exposed to monoclonal anti-FLAG (α-FLAG), polyclonal anti-CagA (α-CagA), or polyclonal anti-CagF (α-CagF) serum as appropriate. The strains G27 (wild type) and G27/8-FL served as negative controls to test for proteins that might unspecifically bind to the beads or the FLAG tag, respectively.

unspecific binding of any of the proteins probed by immunoblotting (Fig. 3A and B.).

Subcellular localization of CagF. We next sought to determine the cellular location of CagF by use of the bacterial fractionation protocol of Gauthier et al. (25). This protocol has been well established for enteropathogenic *E. coli* and yielded a better fractionation than similar protocols used for *H. pylori* (7, 21, 22, 40, 60). Through prediction software analysis, it was suggested that CagF is a 31-kDa protein with a pI of 4.64 (Compute pI/M_w tool; http://ca.expasy.org/tools/pi_tool.html), contains no signal peptide sequence (SignalP v3.0; http://www.cbs.dtu.dk/services/SignalP/), and has one strongly predicted transmembrane domain proximal to the C terminus of the protein (DAS [dense alignment sequence]; http://www.sbc.su.se/~miklos/DAS/) (16, 19, 24, 35). The CagF protein was predicted to localize to the cytoplasm by multiple topographic prediction software. However, the transmembrane domain would suggest that a membrane localization is more likely for this protein than the cytoplasm (8, 23).

As Fig. 4A shows, the CagF antibody bound to the inner membrane and cytoplasmic fractions of strain G27 of *H. pylori*. The control inner membrane antibody directed toward the VirB11 ortholog was strongly reactive to the inner membrane fractions (Fig. 4B). Structural analysis suggests an inner membrane localization of VirB11 in *H. pylori*, which is consistent with models proposed for *A. tumefaciens* that suggest inner membrane anchoring and cytoplasmic exposure of the majority of the protein (11, 44, 58). A monoclonal antibody directed

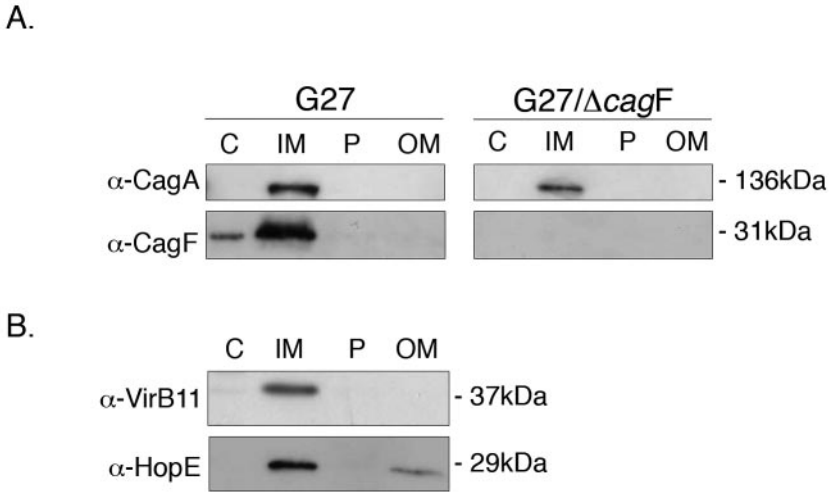

FIG. 4. Bacterial fractionation. G27 (wild type) and G27/Δ*cagF* were fractionated into four major fractions: cytoplasm (C), inner membrane (IM), periplasm (P), and outer membrane (OM). The cytoplasm was purified by ultracentrifugation, the periplasm by lysozyme treatment, and inner and outer membranes by differential solubility in 2% (wt/vol) *N*-lauroyl sarcosyl. Equal percentages of samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. A. Immunodetection of CagF and CagA was conducted by anti-CagF (α-CagF) and anti-CagA (α-CagA) polyclonal sera, respectively. B. Immunoblots were probed with the control antibodies anti-VirB11 (α-VirB11) (inner membrane) and anti-HopE (α-HopE) (outer membrane) to monitor the purity of the fractions.

toward the outer membrane porin HopE was reactive to the outer membrane and the inner membrane (Fig. 4B) (18). These results suggest that the separation of the inner membrane from the outer membrane is not 100% efficient; however, the sarcosine-insoluble outer membrane fraction was free of inner membrane contamination (Fig. 4B). Since the CagF antibody was not reactive against the outer membrane fraction (Fig. 4A), this suggests that CagF is not likely outer membrane localized as previously published. The lack of a signal peptide sequence and the prediction of association with the cytoplasm and inner membrane, combined with our results, all argue against outer membrane localization. It should be noted that the method employed for bacterial fractionation has potential limitations with *H. pylori*. Results may vary depending on the growth phase and exact protocol used. Multiple popular detergent-based separation protocols were conducted, all yielding similar results.

The localization of CagF for bacteria harvested from an AGS tissue culture infection was identical to that for an overnight liquid culture (data not shown), suggesting that host cell contact had no noticeable effect on the localization of CagF. A *virD4* mutant was also used to screen for localization of CagF and CagA. Previous work showed that a mutant of the putative ATP binding and hydrolyzing protein (52) prevents CagA translocation, likely due to a disruption of the T4SS (20). In the absence of the VirD4 protein, CagF and CagA fractionated similarly to the wild type, suggesting that their localization does not depend on the VirD4 protein (data not shown).

CagA localization and stability in a CagF mutant. To investigate a possible function for the CagF-CagA interaction, fractionation experiments were conducted on a Δ*cagF* mutant of G27. The interaction of CagA and CagF paired with the possible inner membrane localization of CagF suggested that CagF might play a role in delivery of CagA to the membrane for transport through the type IV secretion apparatus. When the fractions of G27 and Δ*cagF* were probed with a polyclonal CagA antibody, CagA was found located in the inner membrane for both samples (Fig. 4A). These results suggest that localization of CagA to the inner membrane of the bacterium was independent of CagF. However, since CagF is necessary for the successful translocation of CagA, we tested whether CagF stabilized CagA in *H. pylori*. CagA did not break down in the absence of CagF, suggesting that the stability of CagA in *H. pylori* is not dependent on the interaction with CagF (Fig. 4A). The interaction between CagA and CagF is therefore likely to exert its function after CagA localizes to the membrane through a mechanism still unknown. However, as is visible in Fig. 1, CagA appeared to be more stable when coexpressed with CagF in *E. coli* than CagA expressed alone.

CagF translocation was not detected in AGS cells. To test the possibility that CagF may translocate with CagA, the strains G27, G27Δ*cagF*, G27Δ*virB9*, and G27Δ*cagF*/F-FL were tested in a translocation assay in which bacteria were incubated with AGS cells for 3 h, after which the AGS cells were washed and fractionated into soluble, host membrane, and insoluble fractions by mechanical lysis. This fractionation method was described previously and allows efficient separation of the host cytosol and membrane from the bacteria, which remain in the insoluble fraction. The fractions were analyzed for the presence of CagA, CagF-FLAG, and tyrosine-phosphorylated proteins. In both the wild type and the G27Δ*cagF*/F-FL strain, CagA translocation into the AGS cell membrane was detected by immunoblotting (Fig. 5A), while the G27Δ*cagF* and G27Δ*virB9* strains did not allow for CagA translocation. This result was also confirmed with antiphosphotyrosine antibody, which indicated that translocated CagA (136 kDa) was phosphorylated on tyrosine residues. This process was associated with decreased phosphorylation of host cell proteins (70 kDa) (42, 47). The FLAG antibody was able to detect CagF only in the insoluble fraction containing the bacteria but not in the host cell membrane or the cytosol (Fig. 5A and data not shown). These data suggested that CagF was not translocated with CagA. The requirement of both CagF and VirB9 for CagA translocation was previously reported by Fischer et al. (20). However, in that work the *cagF* mutant was created by insertion of a kanamycin resistance cassette into the *cagF* sequence, and thus, a polar effect on other genes could not be excluded. Our experiment employed a nonpolar in-frame deletion of most of the open reading frame of CagF, which we could use to verify the importance of CagF for CagA translocation. Reintroduction of the *cagF*-FLAG gene into the G27Δ*cagF* strain also demonstrated that CagF-FLAG is functional and able to restore the elongation phenotype of AGS cells (Fig. 5B).

DISCUSSION

The specific mechanism by which *H. pylori* translocates CagA into the host gastric epithelial cell is largely unknown. To date no confirmed interactions between the components of the *cag* T4SS have been reported; however, two recent interactions were detected between *cag* proteins and other *H. pylori* proteins of unknown function. Terradot et al. performed yeast two-hybrid analysis on a subset of gene products, including several *cag* and non-*cag* proteins. Their analysis identified an interaction between the VirB11 ortholog and HP1451 as well as CagA and HP0496 (53). They suggested that *cag-cag* interactions are not easily detected with two-hybrid methods, likely due to their limitation in analyzing interactions of membrane proteins.

CagA is the only known effector protein of the *H. pylori* T4SS. Therefore, we were especially interested to determine which *cag* proteins interact with CagA and to define their role in substrate secretion and translocation. We employed immunoprecipitations to successfully derive the first known interaction between two *cag* proteins, CagA and CagF, both in a surrogate *E. coli* host and in a natural *H. pylori* host. The interaction between CagF and CagA is particularly interesting in *E. coli*, because it shows that the two proteins interact with each other directly without intermediary Cag proteins. Furthermore, the results showed that two CagA species were specifically pulled down. The larger band corresponded to the full-length 135-kDa CagA protein, and the second corresponded to the 100-kDa carboxy-terminally processed CagA protein (32). The latter fragment coprecipitated with CagF, indicating that at least one interaction occurs within the amino-terminal portion of CagA. The 35-kDa carboxy-terminal fragment of CagA was not detectable in the lysates, suggesting that it either was not recognized by the polyclonal CagA antibody or was degraded completely. Thus, we cannot rule out that a

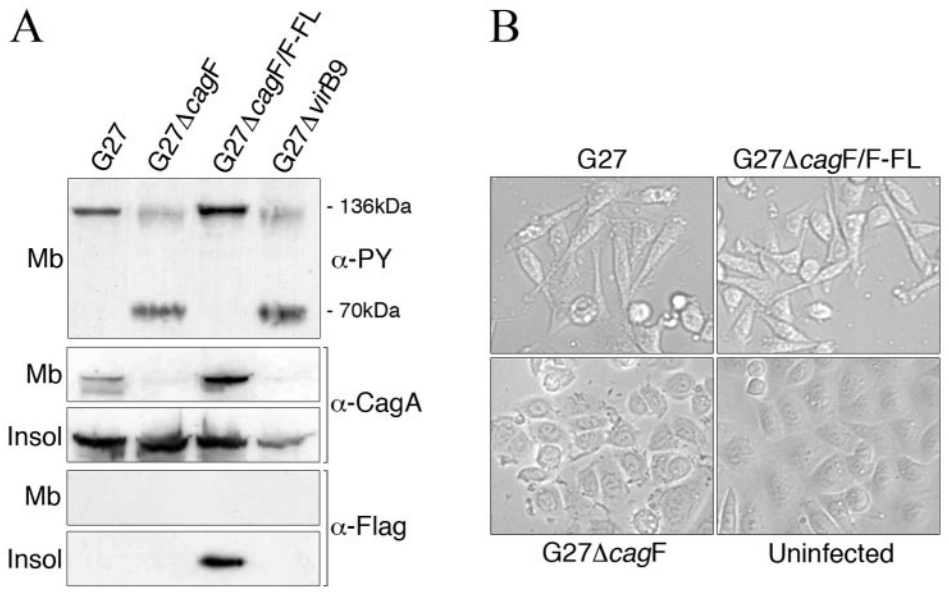

FIG. 5. CagF is required for CagA host cell translocation but is not translocated itself. AGS cells were infected with the wild-type strain G27, its isogenic mutants G27Δ*hp*532 (*virB9*) and G27Δ*cagF*, and G27Δ*cagF*/F-FL for 4 h or left uninfected. (A) Cells were fractionated into membrane (Mb) and insoluble (Insol) fractions as described previously (52). The fractions were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were probed with anti-CagA (α-CagA), anti-phosphotyrosine (α-PY), or anti-FLAG (α-FLAG) antibodies to screen for CagA translocation, tyrosine phosphorylation, or the CagF-FLAG location, respectively. (B) Cells were visualized by phase-contrast microscopy to verify AGS cell morphology. CagA translocation was indicated by the characteristic elongation phenotype.

further interaction region may indeed exist in the carboxy-terminal 35-kDa region. Experiments to further specify the interacting domains of CagA and CagF are under investigation in our laboratory. However, our preliminary findings have implications for the CagA secretion process. The fact that the CagA-CagF interaction is crucial for CagA translocation into AGS cells combined with the finding that an interaction occurs within the amino-terminal 100-kDa region suggest that this region encodes at least one CagA secretion and/or translocation motif. Interestingly, in type IV secretion systems of *A. tumefaciens* (VirF and VirE2) and *Legionella pneumophila* (RalF), the extreme C terminus was sufficient for secretion of reporter fusions (5, 34, 45, 56). However, the Beps substrates of *Bartonella henselae* serve as another example where a second protein domain was required in addition to the C-terminal signal (45).

Another important finding of our work is that besides its major interaction with CagA, Cag-F-FLAG also appeared to interact with additional proteins. These were not investigated in this work, but it is likely they are indicative of the remaining proteins of a subcomplex that possibly involves additional inner membrane-associated *cag* proteins. Additional interactions independent of CagF should also be expected for CagA during its passage through the periplasm and the outer membrane. It is likely that each protein interacts with several proteins independently of the other partner. In this sense, the proteins may interact at different times and locations, as has been recently documented for the VirB/D4 system in *A. tumefaciens* (12). Determining when and where CagA and CagF interact will be of particular interest in defining the role of this interaction in effector secretion.

We demonstrated that CagA was located in the bacterial inner membrane fraction, independent of CagF. This would suggest that CagA does not rely on CagF to locate to the membrane; however, it is possible that CagF acts as a modified chaperone-like protein by binding CagA once at the membrane and delivering it into the actual secretion apparatus. This would assign a chaperone-like function to CagF that is based not on a cytoplasmic association but rather on subsequent trafficking to the secretion complex. The theoretical pI of CagF was estimated to be 4.64, the lowest for the putative proteins in the *cag* PAI. Chaperone proteins are typically characterized by a low pI and molecular weight (39). Though the pI of CagF was consistent with a chaperone, the molecular weight was larger than those of most known chaperones from type three secretion systems (39). Our results suggesting localization to the cytoplasm and the inner membrane would be in agreement with our proposed modified chaperone-like function. Furthermore, CagF was not translocated into AGS cells with CagA, suggesting that CagF is in fact either a chaperone-like protein or a structural inner membrane component of the *H. pylori* T4SS that may be involved in the first steps of substrate recognition.

In conclusion, we report the first documented interaction between CagA and another *cag* protein and thus the first direct interaction within the *H. pylori cag* T4SS. Further work is required to define the role of CagF in substrate recognition and secretion by the *H. pylori* T4SS.

ACKNOWLEDGMENTS

We thank James Gunton and Zaher Zeaiter for invaluable scientific discussion. We also thank Wolfgang Fischer for the Hp0525 (VirB11) antibody. Furthermore, we thank Peter Doig for supplying us with the HopE antibody and Sarah Keen for technical assistance. For reagents,

including *Helicobacter pylori* strain G27, we greatly thank Antonello Covacci. And finally, we thank Mark Peppler for his critical review of the manuscript.

This work was supported by a Canadian Institutes of Health Research (CIHR) operating grant (MOP-62779) and an Alberta Heritage Foundation for Medical Research (AHFMR) establishment grant (200200583) to M.S. We are also thankful for the support received by the Canada Foundation of Innovation (CFI) and the Alberta Science and Research Investments Program (ASRIP). M.S. is an AHFMR Research Scholar.

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Editor: D. L. Burns